Samantha A. Yost, April R. Giles, William M. Henry, Karolina J. Janczura, Justin Glenn, Devin S. McDougald,
Kirk Elliot, Randolph Qian, Subha KarumuthilMelethil, Ye Liu, Olivier Danos, Andrew C. Mercer
Gene Transfer Technologies, REGENXBIO, Inc., Rockville, MD

Introduction: The field of AAV gene therapy is quickly growing from treating small numbers of patients with rare diseases to more common clinical indications that affect larger populations. However, the literature on the effects of sexual dimorphism and genetic background on AAV transduction is limited to a small number of serotypes. In this study, we sought to characterize the tissue transduction properties of 118 pooled AAV capsids in male and female mice as well as male *mdx* mice (C57BL/10ScSn-*DMD*^{mdx}/J), a common model for Duchenne muscular dystrophy. **Results**: The AAV capsid pool consisted of 118 vectors, comprising both naturally occurring serotypes and engineered variants, with a barcoded CAG-GFP transgene. The AAV pool was administered by intravenous injection at a dose of 1.77 x 10^{13} GC/kg to female C57BL/6 (n=5), male C57BL/6 (n=5), and male *mdx* (n=5) mice, all six to eight weeks old. Vector genome copies (GC) per diploid cell and transcripts per µg RNA were quantified by digital PCR with primers for GFP and mouse glucagon as an endogenous genome control. Next generation sequencing was used to assess the relative transduction of all 118 capsids. Our results indicate that transduction differences by sex are tissue-specific and similar trends were observed across all AAV capsids and clades. In muscle tissues, we saw a 9-fold increase in GC/cell in the heart and 2-fold increase in gastrocnemius in female compared to male mice. By contrast, in liver we found a 2-fold increase in GC/cell in male compared to female mice. Male C57BL/6 mice had higher GC/cell in muscle tissues, including gastrocnemius, tibialis anterior, triceps, and diaphragm, compared to *mdx* mice. Surprisingly, this trend was highly reversed at the RNA expression level with a 24fold increase in transcripts/µg RNA in *mdx* tibialis anterior, 8-fold in *mdx* gastrocnemius, and 2-fold in *mdx* heart compared to C57BL/6 male mice. In liver, both male C57BL/6 and *mdx* mice had similar GC/cell; however, C57BL/6 male mice had 3-fold higher transcripts/ µg RNA compared to *mdx* mice. **Conclusions**: Differences in AAV gene transfer efficiency by sex or genetic background were found to be tissue dependent. Furthermore, trends in vector genome quantity and RNA expression level were not always concordant. Thus, both sex and genetic background of animal models should be considered in the development of AAV gene therapy.

95. High Throughput Screening of Diverse Mini-Promoter Libraries within AAV via Expression Linked Promoter Selection (ELiPS)

Kazuomori K. Lewis[1], Joost van Haasteren[1], David V. Schaffer[2]
[1] Bioengineering, UC Berkeley, Berkeley, CA,[2]Chemical and Biomolecular Engineering, Bioengineering, and Neuroscience, UC Berkeley, Berkeley, CA

The limited carrying capacity of the AAV genome (4.7 kb) necessitates the careful design of transgenes to maximize gene therapy efficacy and safety. A major design component of the transgene is the promoter, which dictates when, where, and how much of a therapeutic payload is expressed. Some applications require strong, ubiquitous expression, yet the common constitutive promoters - such as CMV (>750 bp) and CAG (>1600 bp) - are moderately sized and thus place limitations on the rest of the vector genome (e.g. Cas9). Truncated versions of these promoters are typically significantly weaker. Other applications call for cell or tissue specific expression, yet available specific promoters tend to be large and weaker in expression than constitutive promoters. We have developed a platform for the efficient generation of large (>10^7) libraries of synthetic promoters that can be functionally screened using AAV vectors for the high throughput selection of promoters based on their expression properties in cells or tissues of interest. We initially focused on engineering small (~200 bp), strong ubiquitous promoters for use in AAV-mediated gene delivery. To date, the largest screen of synthetic promoters in mammalian cells has been a microprinted array of 52,000 library members. We have developed a method termed ELiPS (Expression Linked Promoter Selection) in which synthetic promoters are built sequentially from small motifs in coordinated steps, allowing precise control of promoter size. ELiPS enables the construction of synthetic promoter libraries in which a barcode in the 3` UTR of the mRNA transcript is directly linked to the identity of the promoter that drove its expression, a design that is amenable to next generation sequencing analysis of promoter strength. Based on bioinformatic analysis of gene expression databases, we engineered two large libraries containing over 5x10^7 short (~200 bp) synthetic promoters intended for ubiquitous, AAV-mediated gene expression. Through just a single round of selection in an initial experiment in HEK 293T cells, we have identified a synthetic promoter of 218 bp termed ELiPS-L2-293T.1 (EL2T.1) that has 76% of the activity of the CAG promoter (1664 bp) and 82% of the activity of the CMV promoter (808 bp) - via flow cytometry, MFI_{CAG} 8570 ± 611, MFI_{CMV} 7985 ± 1128, $MFI_{EL2T.1}$ 6583 ± 1118. The expression level of EL2T is also not statistically significantly different from that of CMV ($p = 0.159$, twotailed Student's t-test, unequal variance). This approach is currently being applied to screen for small, strong, constitutive promoters in human tissue models. Our ELiPS platform is thus highly effective in the generation and high throughput screening of large synthetic promoter libraries. We are now in the process of applying our platform technology to identify strong, short promoters in human *in vitro* culture models and furthermore anticipate its translation *in vivo* to derive tissue and cell-specific promoters.

96. GMEB2 is a Conserved Cellular AAV Restriction Factor That Inhibits Transduction of Human Stem Cells

Amanda M. Dudek, Nicole M. Johnston, Sriram Vaidyanathan, Sridhar Selvaraj, Matthew H. Porteus
Pediatrics, Stanford University, Palo Alto, CA

Cellular factors that inhibit AAV transduction are of great interest to identify areas of improvement for AAV-based gene therapies for both episomal AAV expression as well as AAV-mediated genome editing. Here, we have identified the P79 component of the parvovirus initiation factor (PIF) complex, GMEB2, as a highly conserved AAV restriction factor. Knock-out of GMEB2 increases transduction in cell lines and primary cells across all tested AAV capsids including AAV1, AAV2, AAV4, AAV5, AAV6, AAV7, AAV9, and AAVDJ and at both high and low genome copy number, demonstrated by an up to 10-fold increase in GFP positive cells. At high transduction levels (above 90% GFP+ in WT cells), MFI is drastically increased demonstrating a potential of GMEB2 modulation to increase not only overall number of transduced cells, but also expression level within the cell. Increased transduction in GMEB2 KO cells is observed for constructs with a variety of promoters, such as CMV and UBC demonstrating that this restriction is specific to the AAV sequence. GMEB2 is highly expressed in primary cell types that are inefficiently transduced by AAV including human induced pluripotent stem cells and human airway stem cells. In contrast, CD4+ T cells and hematopoietic stem and progenitor cells (HSPCs) which are more efficiently transduced by AAV express little or no detectible GMEB2. Using a highly efficient multiplexed guide RNA/Cas9 strategy for knock-out, we demonstrate that loss of GMEB2 increases transduction in clinically relevant primary human airway bronchial stem cells which we have previously used for AAV-mediated genome editing as a corrective therapy for Cystic Fibrosis. Subcellular fractionation and western blot experiments demonstrate that although GMEB2 is reported to be a DNA-binding protein, restrictive cell types express high amounts of cytoplasmic and/or membrane associated GMEB2 rather than nuclear-localized GMEB2. Droplet digital PCR from knock-out cell fractions transduced with AAV demonstrate an increase in overall genome number in the lysates, while maintaining the overall genome distribution in cytoplasmic vs. membrane vs. nuclear fractions, suggesting that the restrictive effect of GMEB2 occurs at an early stage in the entry pathway. Additionally, in the presence of nucleofection the beneficial effect of GMEB2 KO is lost, suggesting that nucleofection at the time of AAV transduction may bypass the restrictive activity of GMEB2. These studies demonstrate a highly conserved AAV restriction factor which is capsid independent and influences the transduction efficiency of primary human stem cells.

97. Rationally Designed Inverted Terminal Repeats Improve AAV Vector Production

Liujiang Song[1,2], Zhenwei Song[1], R. Jude Samulski[1,3], Matthew L. Hirsch[1,2]

[1]Gene Therapy Center, University of North Carolina, Chapel Hill, NC,[2]Department of Ophthalmology, University of North Carolina, Chapel Hill, NC,[3]Department of Pharmacology, University of North Carolina, Chapel Hill, NC

Gene delivery approaches using Adeno-associated virus vector (AAV) are currently the leading method of human gene therapy. In all clinical AAV gene therapy applications, the only conserved viral DNA sequences in recombinant AAV (rAAV) are based on the inverted terminal repeats of serotype 2 (ITR2), which are required for multiple aspects of the AAV life cycle such as genome replication and encapsidation. Our laboratory has engineered a panel of functional synthetic ITRs (synITRs) via rational nucleotide substitutions and deletions of putative host factor binding sites. To assess the influence of ITR sequence variations, transgenic genomes flanked by ITR2 or synITRs were evaluated for rAAV production. Additionally, several biological characteristics were analyzed including: 1) the impact of the ITR sequences on rAAV genome replication, 2) the packaging efficiency and genomic integrity, and 3) overall rAAV titers. This study

also considered the impact of the synITR sequences on inherent ITR transcriptional activity and exogenous promoter function following rAAV transduction. Derived data demonstrated a phenomenon of ITR sequence-dependency on rAAV yield, as well as the capsid:genome packaging ratio. Notably, vectors exhibiting synITR257, an ITR2 size-matched sequence in which selected guanine-cytosine rich regions were substituted with adenine-thymine nucleotides, showed a significant three-fold increase in vector production compared to ITR2-based rAAV as determined by probe-based qPCR, supported by alkaline gel electrophoresis, and capsid packaged ITR sequencing. The hypothesis of increased transgenic genome replication to account for the increased rAAV yield was refuted as results indicate that vector genomes employing synITR257 replicate less efficiently than ITR2flanked genomes in production cells. This is the first evidence showing that transgenic genome replication is not a rate-limiting step for rAAV production using 293 triple transfection production methodology. Interestingly, synITR257 significantly enhanced the encapsidation efficiency of rAAV vectors determined by the capsid:genome ratio compared to ITR2. Furthermore, preliminary results suggest that the synITRs and ITR2 may have different inherent transcriptional activity following transduction with a promoter-less luciferase transgene cassette. However, in the context of a strong exogenous promoter, reporter activity was not significantly different between synITR257 and ITR2 following transduction of the evaluated cell lines. The collective data demonstrate that rAAV transgenic genome replication is not rate-limiting for rAAV production and that modulation of ITR sequences influence rAAV yield, potency, and thus represents another optimization step for enhanced and potentially safer AAV-based gene therapy drugs.

98. Characterization of AAV Inverted Terminal Repeats by Atomic Force Microscopy

Marianne Laugel*[1], Yeraldinne Carrasco-Salas*[2], Blandine Simon[2], Mathieu Mevel[1], Oumeya Adjali[1], Cendrine Moskalenko#[2], Magalie Penaud-Budloo#[1]

[1]INSERM UMR 1089, University of Nantes, Nantes, France,[2]UMR 5672, ENS de Lyon, Lyon, France

The rational design of a recombinant adeno-associated vector (rAAV) consists of removing the three open reading frames of the wild-type AAV and cloning a sequence of interest between the two inverted terminal repeats (ITR). Hence, ITRs are the only viral elements kept in the AAV vector genome. AAV ITRs are 143-167 nucleotides in length. These viral telomeres are composed of

imperfect palindromic regions folding into a particular T-shaped structure. Essential for vector production, they contain the minimal elements required *in cis*for rAAV genome replication and encapsidation. With the numerous successes of gene therapy clinical trials and the commercialization of AAV-based drugs, there is a need for better understanding and characterizing these vectors. In particular, ITRs have been shown to be involved in cell-specific transcription, leading to renew interest in these fascinating sequences. In this work, we have developed a protocol allowing the visualization of AAV vector genomes by Atomic Force Microscopy (AFM), and specifically the assessment of ITR secondary structures. Single-stranded genomes flanked by wild-type AAV2 ITR were extracted from AAV vectors, incubated with Mg2+ and absorbed on a Mica surface for AFM imaging in air and in Peak Force mode. Length

and height of observed molecules were calculated using Matlab and Gwyddion programs. A majority of intact full-length rAAV genomes of approximatively 1.5 µm (4.5kb) were observed, validating our DNA extraction method. The presence of folded ITRs at the extremities of the vector genome was confirmed by height measurement. Moreover, three types of ITR secondary structures were found: a T-shape structure consistent with the most energetically stable structure predicted by RNAfold, a linear form consistent with inter-molecular annealing of unfolded ITRs and an intermediate folding conformation. Our study confirms that ITRs are dynamic structures that can adopt diverse folding structures. Controlling experimental parameters, our method could give new insights into the secondary structures of rAAV DNA, and particularly of ITRs, in different cellular compartments. Further investigations will be necessary to determine how these secondary structures could impact the rAAV molecular fate and DNA-triggered cellular responses.

Preclinical Gene Therapy for Neurologic Disorders II

99. A Novel Exon Specific U1 snRNA Therapeutic Strategy to Prevent Retinal Degeneration in Familial Dysautonomia

Anil Chekuri[1,2], Elisabetta Morini[3], Emily Logan[1], Aram Krauson[1], Monica Salani[1], Giulia Romano[4], Federico Riccardi[4], Franco Pagani[4], Luk H. Vandenberghe[4], Susan A. Slaugenhaupt[3]

[1]Center for Genomic Medicine, Massachusetts General Hospital Research Institute, Boston, MA,[2]Department of Neurology, Harvard Medical School, Boston, MA,[3]Department of Neurology, Harvard Medical School, Center for Genomic Medicine, Massachusetts General Hospital Research Institute, Boston, MA,[4]Grousbeck Gene Therapy Center, Schepens Eye Research Institute and Massachusetts Eye and Ear Infirmary, Boston, MA

Familial dysautonomia (FD) is an autosomal recessive neurodegenerative disorder caused by a splice mutation in the gene encoding Elongator complex protein 1 (*ELP1*, also known as *IKBKAP*). A T-to-C base change in the 5' splice site of *ELP1* exon 20 results in exon 20 skipping with tissue specific reduction of ELP1 protein predominantly in central and peripheral nervous system. In addition to complex neurological phenotype, FD patients also exhibit progressive retinal degeneration severely affecting their quality of life. To test novel splicing-targeted therapeutic approaches, we developed a phenotypic mouse model of FD, *TgFD9; Ikbkap*$^{20/flox}$ which exhibits most of clinical features of the disease while displaying the same tissue specific mis-splicing observed in patients. Here, we report a thorough characterization of the retinas of our FD mouse using SD-OCT and immunohistochemical assays during disease progression. Our findings showed a significant decrease in the thickness of the retinal nerve fiber layer (RNFL) and the ganglion cell layer (GCL) starting from 3 months of age. Retinal whole-mount analysis showed reduction of RGC cell counts from 6 months of age. Histopathological analysis of the optic nerve from FD mice using neurofilament (NF) staining indicated diffuse degeneration of axonic bundles demonstrating that our mouse model correctly recapitulates the retinal degeneration observed in patients. To restore correct *ELP1* splicing defect and rescue retinal degeneration, we have designed a novel splice targeted therapy using modified version of the spliceosomal U1 snRNAs (ExSpeU1s) that permit targeted binding to intronic sequences downstream of the mutant 5' splice site enabling efficient recruitment of spliceosomal machinery. We have analyzed the efficiency of splicing correction of the ExSpeU1s through *in vivo* delivery using adeno associated vectors (AAV). Our findings suggest that our novel FD mouse model exhibit most of retinal degeneration pathology observed in FD patients. Our *in vivo* preliminary data demonstrate the valuable therapeutic potential of ExSpeU1 RNA delivery to treat retinal degeneration in FD.

100. Novel RNA-Targeting Gene Therapy Approach for Usher's Syndrome Type II Retinitis Pigmentosa

Daniel Gibbs, Rea Lardelli, Greg Nacthrab, Daniela M. Roth, Shawn Lee, Claire Geddes, Alistair Wilson, Nandini Narayan, Dimitrios Zisoulis, Ranjan Batra

Locanabio, Inc., San Diego, CA

Mutations in the USH2A gene are the most common cause of Usher Syndrome type II (USH2) and non-syndromic autosomal recessive retinitis pigmentosa (10-15% of ARRP). Mutations in exon 13 of USH2A gene are the most common mutations in USH2A gene (~16,000 patients in the North America and EU) and lead to loss of function of the very large (600KD) Usherin (USH2A) protein. USH2A isoforms lacking exon 13 lead to the production of a slightly truncated functional protein. Recently, genome-editing and antisense oligonucleotide (ASO)-based approaches have been described to target USH2A exon 13 at the DNA and RNA levels, respectively. Antisense oligonucleotides require recurrent delivery in the intravitreal space and DNA targeting entails the risk of permanent DNA-level off-target effects. Here we describe an RNA-targeting CRISPR/Cas13d gene therapy approach for skipping USH2A exon

13. Our *in vitro* data demonstrates that our candidate shows >98% efficacy in skipping exon 13 (alternative splicing) leading to production of the slightly shortened but functional Usherin protein. Furthermore, we packaged this candidate in a single AAV gene therapy delivery vector for one-time treatment. We screened various AAV serotypes including but not limited to AAV2, AAV5, AAV7, AAV8 and AAVrh10 to optimize delivery to the photoreceptors in wild-type mice. We also constructed another version of our potential therapeutic to skip mouse exon 12 (equivalent to the human exon 13) that requires two guide RNAs along with dCas13d, which shows dose-dependent and highly efficient exon 12 skipping *in vitro*. Finally, we show photoreceptor transduction, safety (4 and 8 weeks), and molecular efficacy (exon 12 skipping) of our AAV-packaged USH2A therapeutic candidate post sub-retinal delivery *in vivo* in mice. These findings support the development of RNA-targeting gene therapies using highly efficacious CRISPR/Cas13d exon skipping in USH2A for patients with exon 13 mutations.

101. Evolution of Modified AAV Vectors in Rhesus Macaque Cochlea

Paul T. Ranum[1], Stephen R. Chorney[2], Yong Hong Chen[1], Megan S. Keiser[1], Xueyuan Liu[1], Congsheng Chen[1], Geary Smith[1], Amy Muehlmatt[1], Luis Tecedor[1], Robert C. O'Reilly[2], Beverly L. Davidson[1,3]

[1]Center for Cellular and Molecular Therapeutics, The Children's Hospital of Philadelphia, Philadelphia, PA,[2]Division of Pediatric Otolaryngology, The Children's Hospital of Philadelphia, Philadelphia, PA,[3]Department of Pathology & Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Introduction: Gene therapy is a powerful tool to combat hearing loss and deafness, but its clinical implementation is impeded by the temporal bone anatomy, a high diversity of genetic lesions, and myriad cochlear sensory- and supporting-cell types. The identification of adeno-associated virus (AAV) capsids capable of efficient hair-cell transduction, including Anc80L65 and AAV2, has brought cochlear gene therapy closer to a clinical reality for some forms of genetic deafness. However, for genetic lesions impacting hearing at the level of cochlear supporting cells, additional vectors with varying tropism characteristics are needed. To meet this need we implemented a directed AAV evolution screen in the nonhuman primate (NHP) cochlea to identify a library of AAV capsid variants with diverse transduction properties capable of satisfying the requirements of current and future cochlear gene therapy designs. **Methods:** Peptide modified AAV libraries were first generated by insertion of random peptides into AAV1, AAV2, and AAV9. In vivo selection was performed in two successive enrichment steps in which our AAV capsid library was delivered to tissues of interest. Tissues were then recovered and capsid variants were extracted and used as the basis for the subsequent round of in vivo enrichment. At each round of enrichment NGS amplicon sequencing libraries were generated and sequencing data was used to track enrichment of each capsid configuration. Initially, our AAV capsid library was delivered to the NHP brain by Intracerebroventricular (ICV) injection. In this experiment we observed striking and unexpected transduction of NHP cochlear hair-cells and supporting cells by AAV9 derived capsid variant. This unexpected transduction of cochlear cells following ICV injection may occur via the fluid connection of CSF to the perilymph bathing the cochlear aqueduct. This finding prompted us to test direct injection of the AAV9 variant by round window membrane injection with lateral canal fenestration (RWM+LCF) and to undertake a separate cochlea-specific AAV enrichment study in which we delivered our AAV capsid library directly to the cochlea via the same RWM+LCF method. One cochlea received direct injection of 1999 and four cochlea (two NHPs) received injections of the AAV capsid library over the course of two rounds of in vivo enrichment. **Results:** Here we report the successful delivery and selection of modified AAV vectors in the cochlea of Rhesus macaque. Using a fluorescent reporter, we observed that the AAV9 variant transduces cochlear inner hair cells effectively, though incompletely, as well as an assortment of supporting cells including cells of the stria-vascularis and spiral ganglion neurons. Sequencing results from the directed cochlear AAV evolution strategy reveal an assortment of enriched capsid variants for which small-library- and fluorescence-based validation is ongoing. Given the remarkable similarity with the human cochlea, the

Rhesus macaque animal model provides a tremendous opportunity to refine both delivery methods and identify AAV capsid variants with high potential for clinical applicability.

102. A Novel Retinal Gene Therapy Strategy for Batten Disease and Beyond

Maura Schwartz[1], Archana Jalligampala[2], Alex Campbell[3], Isabella Palazzo[3], Shibi Likhite[1], Andrew Fischer[3], Maureen McCall[2], Kathrin Meyer[1]

[1]Center for Gene Therapy, The Research Institute at Nationwide Children's Hospital, Columbus, OH,[2]Kentucky Lions Eye Institute, Louisville, KY,[3]The Ohio State University, Columbus, OH

Batten Disease is a fatal, lysosomal storage disorder characterized by cognitive and motor deficits, vision impairments, and seizures. Loss of vision is a characteristic in 10 of the 13 Batten Disease subtypes. Our group has pioneered AAV9 gene therapy treatments that achieve widespread transduction of the brain and spinal cord. However, based on non-human primate data, AAV9 transduction of the retina after CSF delivery might be suboptimal. Thus, strategies to further improve retinal targeting using the same AAV9 gene therapy vector already in clinical trial could further improve Batten Disease gene therapy. Similar to most genetic ocular diseases, photoreceptor degeneration is the most commonly cited pathology in patients. However, recent studies suggest that in some subtypes of Batten Disease such as CLN3 and CLN6, expression must be rescued within cells of the inner nuclear layer (INL) of the retina to prevent vision loss. However, a full visual rescue was not achieved in mouse studies with INL targeting alone. We performed single-cell RNA sequencing in non-human primates to confirm CLN gene expression and found a generally ubiquitous, low expression in almost all cell-types of the retina. This suggests the need for a wide-ranging retina-tropic AAV that efficiently targets the INL, among other cell-types, which currently does not exist. We have recently discovered that administration of neuraminidase (NA), a sialidase enzyme, prior to or in combination with AAV9. GFP, drastically increases

transduction through all the retinal layers in the murine and porcine retina. In mice, we delivered 11 mU of Neuraminidase prior to or in combination with $2e^{10}$vg AAV9.GFP. This manipulation resulted in an increase of GFP+ cells in the murine inner retina. Hand counts of GFP+ cells co-localized with cell-specific counterstains confirmed a 40% increase in AAV9 transduction in Müller glia, a 15% increase in amacrine cells, a 30% increase in bipolar cells, and a 78% increase in horizontal cells. Moreover, we translated this promising treatment paradigm to a WT porcine model. Intravitreal injections delivered 844mU of Neuraminidase prior to or in combination with $2e^{12}$ vg AAV9.GFP. Confocal imaging of retinal sections shows GFP expression in almost all, if not all, retinal cell types. This suggests that it may be possible to target every cell-type of the retina with a single AAV vector. This is especially important with respect to the treatment of Batten Disease and has many additional implications. Also, this strategy would be highly useful in the field of optogenetics, where there is a need for an efficient method to express light-sensitive opsins in cells of the INL to restore sight in individuals that have already lost their photoreceptors.

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103. Reprogramming to Recover Youthful Epigenetic Information and Restore Vision

Yuancheng Lu[1], Benedikt Brommer[1], Xiao Tian[1], Anitha Krishnan[1], Margarita Meer[1], Chen Wang[1], Noah Davidsohn[1], George Church[1], Konrad Hochedlinger[1], Vadim Gladyshev[1], Steve Horvath[2], Morgan Levine[3], Meredith Gregory-Ksander[1], Bruce Ksander[1], Zhigang He[1], David Sinclair[1]

[1]Harvard Medical School, Boston, MA,[2]Department of Human Genetics, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA,[3]Department of Pathology, Yale School of Medicine, New Haven, CT

Aging is a degenerative process that leads to tissue dysfunction and death. A proposed cause of aging is the accumulation of epigenetic noise that disrupts gene expression patterns, leading to decreases in tissue function and regenerative capacity. Changes to DNA methylation patterns over time form the basis of aging clocks, but whether older individuals retain the information needed to restore these patterns— and, if so, whether this could improve tissue function—is not known. Over time, the central nervous system (CNS) loses function and regenerative capacity. Using the eye as a model CNS tissue, here we show that ectopic expression of Oct4 (also known as Pou5f1), Sox2 and Klf4 genes (OSK) through AAV2 in mouse retinal ganglion cells restores youthful DNA methylation patterns and transcriptomes, promotes axon regeneration after injury, and reverses vision loss in a mouse model of glaucoma and in aged mice. The beneficial effects of OSK-induced reprogramming in axon regeneration and vision require the DNA demethylases TET1 and TET2. These data indicate that mammalian tissues retain a record of youthful epigenetic information— encoded in part by DNA methylation—that can be accessed to improve tissue function and promote regeneration in vivo.

104. Efficacious, Safe, and Stable Inhibition of Corneal Neovascularization with rAAV-*KH902* in a Mouse Model of Corneal Alkali injury

Wenqi Su#[1,2], Shuo Sun#[1,3], Bo Tian#[1], Phillip W. L. Tai[4], Jihye Ko[4], Yongwen Luo[4], Xiao Ke[5], Qiang Zheng[5], Hua Yan*[2], Guangping Gao*[4], Haijiang Lin*[1]

[1]Department of Ophthalmology & Visual Sciences, University of Massachusetts Medical School, Worcester, MA,[2]Ophthalmology, Tianjin Medical University General Hospital, Tianjin, China,[3]Tianjin Key Laboratory of Retinal Fuctions and Diseases, Tianjin International Joint Research and Development Centre of Ophthalmology and Vision Science, Eye Institute and School of Optometry, Tianjin, China,[4]Horae Gene Therapy Center, University of Massachusetts Medical School, Worcester, MA,[5]Chengdu Kanghong Pharmaceuticals Group, Chengdu, China

Introduction: Corneal avascularity is an essential prerequisite of corneal transparency. Pathological conditions such as inflammation, hypoxia, and limbal barrier dysfunction disrupt the balance of pro- and anti-angiogenic factors. Under these conditions, corneal neovascularization (CoNV) can occur, causing a decrease in corneal transparency and visual impairment. CoNV develops in over 1.4 million patients in the United States per year. Among the well-studied anti-VEGF drugs in clinical testing, periodic injections of KH902 (trade name, conbercept) can inhibit ocular neovascularization. However, more efficacious and long-lasting treatment for patients with clinical CoNV is an urgent need. To this end, we investigated the long-acting anti-angiogenesis effects and safety of rAAV-delivered *KH902* in a mouse corneal injury model. **Methods:** In this study, we packaged the *KH902* transgene into rAAV vectors, and delivered them by single intrastromal or subconjunctival injections. The transduction efficiency of vectored *KH902* and its cell tropism in the cornea were determined by immunostaining. Droplet Digital PCR was used to quantitate *KH902* mRNA expression. Potential side effects were evaluated by measuring the central corneal thickness (CCT) at various time points by using optical coherence tomography (OCT). Immunostaining of corneal sections with CD11b and F4/80 was performed to assess immune responses two weeks post rAAV-*KH902* injection. Progression of CoNV in an alkali burn-induced model was tracked and quantified at 5 and 10 days, and 2, 3, 4, 8, and 12 weeks post-administration of rAAV-*KH902* by live animal imaging microscopy. Activation of DLL4/Notch signaling and ERK, downstream of effectors of VEGF signaling, were assessed by immunostaining and Western blot. **Results:** We demonstrate that rAAVs can successfully mediate KH902 expression in corneal keratocytes after intrastromal administration, while subconjunctival administration resulted in poorer transduction of keratocytes. *KH902* mRNA expression in the

[1] Neuroscience, Voyager Therapeutics, Cambridge, MA,[2]Vector Engineering, Voyager Therapeutics, Cambridge, MA,[3]Vector Production, Voyager Therapeutics, Cambridge, MA

cornea reached peak levels at 1-2 weeks and then gradually declined. Nevertheless, *KH902* was still detectable at three months post intrastromal delivery. In addressing the safety of rAAV-*KH902* in the cornea, we found that high doses (1.6×10^{10} vg/eye) of rAAV-*KH902* triggered an intense immune response, while lower doses (8×10^{8} vg/eye) caused a minimal response. Based on this finding, we adopted the lower dose for our therapeutic studies in the corneal alkali-burn model for CoNV. We observed that rAAV-*KH902* conferred a dramatic suppression of CoNV for at least three months post-alkali burn without adverse events. In contrast, we observed that the inhibition of CoNV conferred by a single administration of the manufactured conbercept drug lasted only for 14 days post injection. Furthermore, the therapeutic outcomes produced by rAAV-*KH902* treatment were concomitant with the downregulation of DLL4/Notch signaling and ERK activation in the treated tissues. **Conclusion:** Our study demonstrates the potential and relative safety of rAAV-based, longterm anti-angiogenesis therapy with *KH902* in the treatment of CoNV. # The authors contributed equally to this work. * Corresponding authors.

105. Efficacy of a Vectorized Anti-Tau Antibody Using Systemic Dosing of a Blood Brain Barrier Penetrant AAV Capsid in Mouse Models of Tauopathies

Wencheng Liu[1], Jerrah Holth[1], Maneesha Paranjpe[1], Xiao-Qin Ren[2], Yanqun Shu[2], Giridhar Murlidharan[1], Charlotte Chung[1], Alex Powers[1], Emalee Peterson[1], Abigail Ecker[1], Usman Hameedi[3], Kyle Grant[3], Vinodh Kurella[2], Dillon Kavanagh[2], Omar Khwaja[1], Jay Hou[2], Steve Paul[1], Kelly Bales[1], Todd Carter[1]

Anti-tau immunotherapy is a promising therapy for tauopathies, including Alzheimer's disease (AD), frontotemporal dementia (FTD) and progressive supranuclear palsy (PSP). While passive immunization with anti-tau monoclonal antibodies has been shown by several laboratories to reduce age-dependent tau pathology and neurodegeneration in mouse tauopathy models, these studies have typically used repeated high weekly doses of antibody and reported only moderate reduction of tau pathology. We have previously demonstrated broad distribution and expression of a vectorized anti-tau antibody in the mouse brain using a blood-brain barrier (BBB) penetrant AAV capsid administered intravenously (IV). This gene therapy-based approach has potential advantages, including continuous expression of antibody in the central nervous system (CNS) after a single administration of vector, increased CNS levels of tau antibody relative to passive immunotherapy, and the potential to target intracellular tau aggregates. Here we describe studies characterizing the onset of transduction following dosing with a vectorized tau antibody and its efficacy in mouse models of tauopathy. AAV vectors comprising a novel capsid and a transgene encoding an anti-tau monoclonal antibody were administered by IV bolus to wild type mice. Biodistribution and cellular tropism in the CNS were evaluated by ELISA and (or) immunostaining, and vector genome levels were quantified using digital PCR, resulting in the detection of antibody expression soon after dosing. We then investigated efficacy in reduction of tau pathology in mouse tauopathy models. Treatment with our vectorized antibody resulted in durable antibody expression in the CNS and a corresponding reduction in CNS insoluble pathological tau and neurofibrillary tangles. Our results indicate that systemic dosing of a vectorized anti-tau antibody using a BBB-penetrant AAV capsid results in reduced tau pathology and may represent a new single-dose therapeutic strategy for treating various tauopathie

Synthetic/Molecular Conjugates and Physical Methods for Delivery

106. Combinatorial Modified mRNA Induces Cardiovascular Regeneration Post Muscle Ischemic Injury

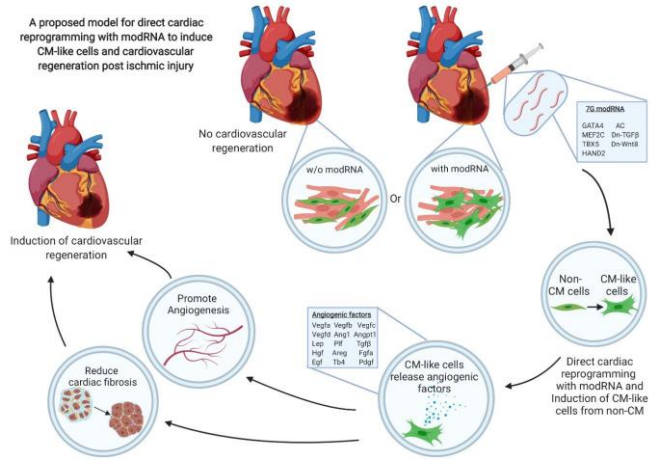
Keerat Kaur[1], Asharee Mahmood[1], Hanna Girard[1], Ann Anu Kurian[1], Magdalena Zak[1], Maria Paola Santini[1], Elena Chepurko[1], Vadim Chepurko[1], Djamel Lebeche[1], Jason C. Kovacic[1], Roger J. Hajjar[2], Shahin Rafii[3], Lior Zangi[1]

[1]Cardiology, Icahn School of Medicine, Mount Sinai, New York, NY,[2]Phospholamban Foundation, Amsterdam, Netherlands,[3]Cardiology, Weill Cornell Medicine, New York, NY

Despite advances in the preventative medicine, ischemic heart disease remains a major cause of morbidity and mortality in the industrialized world, causing significant societal and economic burden. Post myocardial infarction (MI), the adult mammalian heart has limited regenerative capacity to recover from the loss of cardiomyocytes (CM). Thus, reprogramming non-cardiomyocytes (non-CMs) into

cardiomyocyte (CM)-like cells in vivo is a promising strategy for cardiac regeneration. However, the current viral-based gene transfer delivery methods have low and erratic transduction efficiency that precludes these technologies from being translated to the clinic. Modified mRNA (modRNA)-based gene delivery features transient but potent protein translation and low immunogenicity, with minimal risk of insertional mutagenesis. Here we used a modRNA gene delivery platform to deliver different stoichiometry of cardiac-reprogramming genes (Gata4, Mef2c, Tbx5 and Hand2) together with reprogramming helper genes (Dominant Negative (DN)-TGFβ, DN- Wnt8a and Acid ceramidase (AC)), named 7G, to induce direct cardiac reprogramming post MI. In this study, we found that a combination of 7G modRNA cocktail can play a vital role in cardiac reprogramming. Compared to previously established Gata4, Mef2C and Tbx5 (GMT) reprogramming cocktail, 7G modRNA reprogrammed twice the number of nonCMs to CMs (from 28% to 57%) in vitro. Importantly, repeated 7G modRNA transfection results in beating CMs and complete cardiac reprograming *in vitro*. Using our lineage tracing model, we showed that one-time delivery of the 7G-modRNA cocktail at the time of MI partially

reprogrammed ~25% of the non-CMs in the scar area to CM- like cells. Furthermore, 7G modRNA treated mice showed significantly improved cardiac function, longer survival, reduced scar size and greater capillary density than control mice 28 days post-MI. We attributed the improvement in heart function post modRNA delivery of 7G or 7G with increased Hand2 ratio (7G-GMT Hx2) to significant upregulation of 15 key angiogenic factors without any signs of angioma or edema. Intriguingly, 7G modRNA cocktail was also able to induce to neovascularization in mouse hindlimb ischemia model, indicating that 7G-modRNA cocktail administration promotes vascular regeneration in cardiac and skeletal muscle post ischemic injury. Thus, combinatorial, first of its kind 7G modRNA cocktails presents a safe and high efficiency gene delivery approach with therapeutic potential to treat ischemic diseases.



Synthetic/Molecular Conjugates and Physical Methods for Delivery

107. Sustained Episomal Transgene Expression *In Vivo* Driven by Non-Viral DNA Delivery to Rodent Liver

Stoil Dimitrov[1], Joe Sarkis[2], Kevin Smith[3], Ben Geilich[2],
Sean Severt[2], Kevin Davis[4], Edward Acosta[4], Karen Olson[4], Molly Bell[4], Kristine Burke[4], Shan Naidu[4], Eric Jacquinet[4], Carla Leite[2], Andrew Auerbach[2], Nicholas Amato[2], Erik Owen[2], Jordan Santana[2], Tatiana Ketova[2], Brian Fritz[2], Christopher Tunkey[2], Bhargav Tilak[2], Jeffrey Pimentel[1], Mychael Nguyen[1], Susanna Canali[1], Jared Iacovelli[1], Eric Huang[1]

[1]New Venture Labs, Moderna, Cambridge, MA,[2]Platform Research, Moderna, Cambridge, MA,[3]Process Development, Moderna, Cambridge, MA,[4]Non-Clinical Sciences, Moderna, Cambridge, MA

Efficient non-viral delivery of exogenous DNA vectors *in vivo* has the advantage of decreased immunogenicity in comparison to current virally-mediated delivery approaches and largely increased payload capacity. The increased maximal size of the delivered exogenous DNA allows for the introduction of complex transcription units containing the gene of interest together with endogenous or tissuespecific promoter and enhancers, as well as additional mammalian transcription or stability control elements. Here we describe a successful non-viral, Lipid nanoparticles (LNP)-mediated delivery of non-integrating plasmid DNA vectors *in vivo* that drive hepatocytesspecific expression in rodents and allow for long-term stable episomal expression of a transgene. Plasmid DNA-mediated delivery of Firefly Luciferase gene, under control of the liver-specific Alpha-1 Antitrypsin (AAT) promoter, resulted in stable, steady-state Luc expression in mouse hepatocytes for six weeks until study termination. LNPmediated delivery of plasmid DNA encoding a secreted Fc fragment of human IgG1, driven by the liver-specific AAT promoter, resulted in sustained, dose-dependent expression of the secreted hIgG-Fc protein that lasted for six months in Sprague-Dawley rats. Importantly, plasmid DNA-mediated liver-specific transgene expression resulted in mild, transient elevation of ALT and AST six hours after dosing, which resolved by 48h post-delivery. No other changes in the comprehensive blood biochemistry profile of the test subjects were detected. We have observed transient, dose-dependent activation of Type I interferon response after plasmid DNA delivery *in vivo*. In both mouse and rat serum, levels of IFN-alpha, IP-10, MCP-1, MCP-3 and RANTES were transiently elevated at 6h post-delivery and decreased 24h to 48h post dosing. In conclusion, we have demonstrated that LNP-mediated delivery of DNA vectors *in vivo* can lead to long-term, stable tissuespecific expression of both intracellular or secreted proteins in mouse and rat hepatocytes. Expression of transgenes can last for at least 24 weeks. LNP-mediated DNA delivery resulted in mild and transient elevation of liver enzymes and serum inflammatory cytokines.

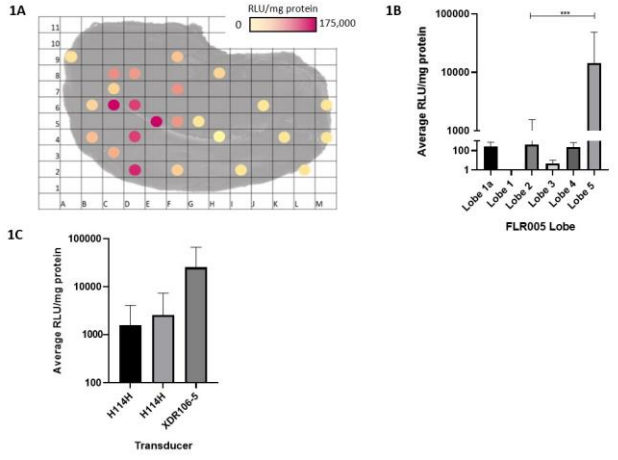
108. Optimization of Transcutaneous Ultrasound Mediated Gene Delivery into Large Animals

Megan A. Manson[1], Feng Zhang[1], Carley M. Campbell[1], Peter Kim[1], Kyle P. Morrison[2], Maura Parker[3], Keith
R. Loeb[3], Dominic Tran[1], Masaki Kajimoto[4], Rainier Storb[3], Carol Miao[1]

[1]Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, WA,[2]Sonic Concepts, Inc., Bothell, WA,[3]Fred Hutchinson Cancer Research Center, Seattle, WA,[4]Seattle Children's Research Institute, Seattle, WA

Hemophilia A is an X-linked clotting disorder characterized by a deficiency of functional factor VIII (FVIII) protein. In previous experiments, we performed transcutaneous ultrasound mediated gene delivery (UMGD) of a FVIII plasmid into normal canines with success using the H114H focused transducer. In order to increase the treatment area, we have recently developed a new transducer, XDR106-5. This transducer is a 5-element, unfocused transducer that leverages both PNP output to circumvent tissue attenuation, as well as a larger treatment area for effective gene delivery. The unique 5-element configuration allows the user to easily scan the transducer to cover a greater treatment area as opposed to the H114 focused transducer, which has a small area of focus. In preparation for future experiments in hemophilia A canines, we aimed to optimize the surgical protocol and ultrasound parameters and tested an improved transducer design. A pGL-4 plasmid encoding the luciferase reporter gene was injected in combination with RN18 microbubbles (MBs) into the canine liver lobes through a fluoroscopy-guided balloon

catheter (either a 12 mm x 20 mm or a 14 mm x 20 mm balloon) inserted into the hepatic vein via jugular vein access. Simultaneously, the transcutaneous therapeutic ultrasound, either H114H or XDR106-5, was applied to induce cavitation in the liver using a pulsed US treatment. Blood was collected at 24 hours and assessed for alanine and aspartate aminotransferase levels. Animals were sacrificed on day one, and liver tissue was harvested and divided into 1 cm x 1 cm sections and subjected to luciferase and Bradford assays. Representative sections were selected from each liver lobe and subjected to H&E staining and reviewed by a certified pathologist. Hemophilia A dogs tend to be larger and therefore have larger blood vessels, so we tested a new balloon catheter in a normal canine of comparable size (22.5 kg). The 12 mm x 20 mm balloon catheter was used in lobe 2, while the 14 mm x 20 mm balloon catheter was used in lobe 5. Lobe 5 demonstrated high expression (Figure 1A) and had significantly higher luciferase expression compared to lobe 2 (Figure 1B). Liver histology showed little change in the liver on day one following the procedure, with no significant injury noted. ALT and AST values were elevated, but at a comparable level to canines from previous experiments, and no veterinary concern was noted. Preliminary data from pig experiments showed that the H114H and XDR106-5 transducers induced similar levels of luciferase expression (RLU/mg protein) in individual sections. However, the XDR106-5 transducer allowed a much larger area of the lobe to be treated. This led to a tenfold increase in average RLU/mg protein per lobe: 2089 RLU/mg on average when treated with H114H and 25486 RLU/mg when treated with XDR106-5 (Figure 1C). We are able to successfully optimize the transcutaneous UMGD procedure in canines by adjusting the surgical procedure for larger canines, and by incorporating a new ultrasound transducer which effectively treats a larger proportion of a targeted lobe.



109. Assembling Several mRNA Strands for Facilitating mRNA Delivery with and without Using Carriers

Satoshi Uchida[1,2], Naoto Yoshinaga[3], Kyoko Koji[3], Horacio Cabral[3]

[1]Kyoto Prefectural University of Medicine, Kyoto, Japan,[2]Innovation Center of NanoMedicine (iCONM), Kawasaki Institute of Industrial Promotion, Kawasaki, Japan,[3]The University of Tokyo, Tokyo, Japan

For developing messenger RNA (mRNA) delivery carriers, it is still challenging to prevent enzymatic degradation before reaching target cells. Although complicated design of polymers or lipids has contributed to solve this issue to some extent, use of such novel materials requires tremendous processes before clinical approval. Herein, we propose a simple strategy of modulating mRNA steric structure that improves nuclease resistance and delivery efficiency of naked mRNA and existing mRNA carriers. To prevent nuclease attack to mRNA by steric hindrance, we structured mRNA by preparing nanoassemblies (NAs) from several mRNA strands, which were crosslinked with each other using RNA crosslinkers hybridizing to mRNA. NAs thus prepared had about 8 mRNA strands and diameter below 100 nm on average. NAs showed around 100-fold increase in nuclease stability compared to naïve mRNA in their naked form. Further mechanistic studies of NAs indicated the contribution of their high-order structure, as well as secondary structure, on the improvement of nuclease stability. Interestingly, NAs retained their translational activity despite their high-order structure, which prevented nuclease attack. Förster resonance energy transfer-based observation of NA structure in intracellular environment indicates that NAs were dissociated selectively in intracellular environment through endogenous RNA unwinding mechanisms coupled with 5′ cap dependent translation. Ultimately, NAs improved mRNA introduction efficiency in their naked form after transfection to culture cells and to mouse brain. Next, we studied the potential of NAs to improve functionality of an existing mRNA carrier. Polymeric micelles (PMs) from poly(ethylene glycol) (PEG)-polylysine with a core-shell structure of PEG shell and mRNAcontaining core were selected because polylysine is widely used for

complexing nucleic acids. Furthermore, PMs loading plasmid DNA were used in a clinical trial. Both PMs loading naïve mRNA and NAs showed average size below 100 nm, with almost neutral ζ-potential. Further physicochemical evaluations including transmission electron microscopic observation and analytical ultracentrifuge revealed that PMs loading NAs possessed a shell with denser PEG chains and a core with more tightly packaged mRNA compared to PMs loading naïve mRNA. As a result, use of NAs in PMs boosted mRNA nuclease stability in serum and in blood circulation after intravenous injection to mice compared to PMs loading naïve mRNA. Ultimately, PMs loading NAs yielded efficient mRNA introduction after transfection to culture cells and to mouse brain. Importantly, PMs loading NAs were stably distributed to whole brain after injection to cerebrospinal fluid, leading to efficient protein expression in wide area of mouse brain. Notably, the utility of NA formulation in naked and PM-loaded forms was demonstrated using several types of mRNA. Conclusively, our strategy effectively improves the functionalities of naked mRNA and existing mRNA carriers, just by changing mRNA structure without the use of additional materials, providing a versatile platform for future clinical applications.

110. Development of Hydrodynamics-Based

Gene Therapy for Liver Cancer

Kenya Kamimura[1], Takeshi Yokoo[1], Hiroyuki Abe[1], Masato Ohtsuka[2], Hiromi Miura[2], Hiroshi Nishina[3], Shuji Terai[1]

[1]Niigata University, Niigata, Japan,[2]Tokai University, Isehara, Japan,[3]Tokyo Medical and Dental University, Tokyo, Japan

Liver cancer is one of the leading causes of cancer-related deaths worldwide. The primary liver cancer is mainly hepatocellular carcinoma (HCC) and currently available therapeutic options, including embolization and chemotherapy, are not effective against advanced-stage liver cancer due to its heterogeneity, high risk of recurrence, and drug resistance. Although recent developments in the field of molecular-based strategies shed light on chemotherapy for HCC using molecular targeting agents, these agents also have limitations due to the heterogeneity of HCC. Therefore, it is essential to develop a novel therapy, and in this context, we evaluated the gene therapy for HCC using a diphtheria toxin fragment A (DTA) geneexpressing plasmid using a nonviral hydrodynamics-based procedure in this study. The complementary DNA of DTA was inserted into the pIRES2 plasmid vector containing an internal ribosome entry site. The expression of DTA was regulated by a chicken β-actin promoter and a cytomegalovirus enhancer (pCAG-DTA) or the human alphafetoprotein (AFP) promoter (pAFP-DTA). The antitumor effect of DTA expression in HCC cell lines including HLE, HLF, and Huh7 and AFP promoter selectivity was evaluated *in vitro* by examining HCC cell growth. We then examined the *in vivo* effect and safety of the AFP promoter-selective DTA expression using an HCC mouse model established by the hydrodynamic gene delivery of the yes-associated protein (YAP)-expressing plasmid. Mice with the YAP-expressing plasmid demonstrated an HCC incidence of >80% and expression of AFP protein. First, we confirmed the effect of DTA on the inhibition of protein synthesis by the cells based on the disappearance of tdTomato protein expression and GFP protein expression cotransfected upon the delivery of the DTA plasmid. The proliferation of HCC cell lines was

significantly inhibited to <20% upon DTA expression in HCC cells in an AFP promoter-selective manner. In addition, concentration of AFP in the cell culture medium was significantly inhibited by pAFP-DTA transfection. Moreover, a significant inhibition of HCC incidence of 80% to 10%; and a suppression of the AFP tumor marker from 13,448.2 ± 8787.2 to 193.5 ± 129.5 ng/mL and des-gamma-carboxy prothrombin from 590.6 ± 306.7 to 70.4 ± 37.7 ng/mL were evidenced in mice treated with hydrodynamic gene delivery of DTA within 2 months after YAP gene delivery. Serum biochemical factors revealed improvement of hepatobiliary enzymes after the DTA gene therapy due to the shrinkage of the tumor, and no adverse events were observed. These results indicate, for the first time, the effect and safety of hydrodynamics-based gene delivery of DTA for HCC in an *in vivo* mouse model.

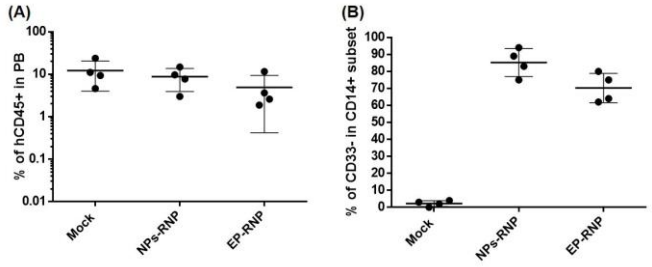
111. Hematopoietic Stem and Progenitor Cells-Targeted Polymeric Nanoparticles for In Vivo Gene Therapy

Rkia El Kharrag[1], Kurt Berckmueller[1], Margaret Cui[1], Ravishankar Madhu[1], Anai Perez[1], Olivier Humbert[1], Hans-Peter Kiem[1,2], Stefan Radtke[1]

[1]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA,[2]Department of Medicine, University of Washington School of Medicine, Seattle, WA

Autologous hematopoietic stem and progenitor cell (HSPC) gene therapy has the potential to permanently cure millions of patients suffering from hematological diseases and disorders. However, current approaches depend on expensive specialized cleanrooms for the manipulation of HSPCs *ex vivo* limiting the feasibility and routine clinical application. Consequently, HSPC gene therapy remains inaccessible to many patients especially in infrastructure poor regions. HSPCs-targeted *in vivo* delivery of gene therapy agents could overcome this bottleneck enhancing the portability of gene therapy by avoiding expensive infrastructure. With the aim to perform HSPCs-targeted gene therapy *in vivo*, we adapted a biodegradable poly (β-amino ester) (PBAE)-based nanoparticle (NP) formulation with targeting ability for the modification of HSPCs. We benchmarked the NP-based delivery approach against our existing electroporation (EP)-mediated CRISPR/Cas9 gene-editing strategy to knockout CD33 on HSPCs and protect them from anti-CD33 targeted acute myeloid leukemia immunotherapy. NPs were generated with Cas9 ribonucleoprotein (RNP) complexes and loaded NPs evaluated for size and charge to correlate physiochemical properties with the outcome, and establish quality control standards. NPs and EP gene-edited $CD34^+$ cells were evaluated phenotypically by flow cytometry and functionally in colony-forming cell (CFC) assays, and in NSG xenograft model. The optimal characteristics for RNP-loaded NPs were determined at 150-250 nm and 25-35 mV. Physiochemical assessment of RNP-loaded NP formations provided an upfront quality control of RNP components detecting degraded components significantly and enhancing the predictability and success of editing. NPs achieved more than 85% CD33 knockout using 3-fold lower dose of CRISPR nucleases compared to EP. No impact on erythromyeloid differentiation potential of gene-edited cells in CFC assays was observed. Most importantly, NP-modified CD34+ cells showed efficient and sustained gene editing in *vivo* with improved long-term multilineage engraftment potential in peripheral blood (PB) and bone marrow stem cells in comparison to EP-edited cells **(Fig. A,B)**. Here, we show that PBAE-NPs enable efficient CRISPR/Cas9 gene editing of human CD34+ cells without compromising the viability and long-term multilineage engraftment of human HSPC *in vivo*. Most importantly, we defined physiochemical properties of PBAE-NPs that enable us to not only determine the integrity of our gene-editing agents, but also predict the efficiency on long-term engrafting HSPCs. The requirement of 3-fold less reagents compared to EP, the ability to lyophilize quality-controlled and ready to administer gene therapy reagents, and the opportunity to engineer the surface of PBAE-NPs with HSPC targeting molecules are the technological foundation for feasible, portable, and safe *in vivo* HSPC gene therapy. Ease of production and versatility of cargo options will hopefully enable the field of *in vivo* gene therapy to make a significant step towards a more accessible and portable gene therapy platform. **Figure. A)** Human CD45+ engraftment and **B)** CD33 knockout percentage in CD14+

subset in PB of mice transplanted with CD33 gene-edited CD34+ cells at week 14 post transplantation.



112. Delivery of CRISPR/Cas9 for Recovering the Expression of the Endogenous FVIII in Hemophilia A Mice

Chun-Yu Chen[1], Xiaohe Cai[1], Carol H. Miao[1,2]

[1]Immunity & Immunotherapies, Seattle Children's Hospital Research Institute, Seattle, WA,[2]Department of Pediatrics, University of Washington, Seattle, WA

Hemophilia A (HemA) is a bleeding disorder resulting from a deficiency of the X-linked factor VIII (FVIII) gene. Currently HemA patients is routinely infused with FVIII protein 3-4 times per week as prophylactic treatment, which is costly and inconvenient. Gene therapy represents a very promising alternative method to treat HemA patients. Advancement of biocompatible nanoparticle (NP) technology enabled delivery of nucleic acids safely into the targeted organs. In combination of CRISPR/Cas9 technique, *in vivo* gene edition to correct the mutant FVIII and regain the expression of FVIII is feasible. We examined the *in vitro* gene delivery efficiency of DNA encapsulated NPs and *in vivo* FVIII gene editing, respectively. Since FVIII protein is mainly and naturally made in liver sinusoidal endothelial cells, we synthesized NPs that can selectively target endothelial cells. DNA encapsulated NPs was synthesized using nanoprecipitation by the combination of an organic phase containing chondroitin sulfate and sorbitan ester and an aqueous phase containing p2X-GFP plasmid DNA. DNA encapsulation efficiency was examined by DNA electrophoresis. By titrating different DNA:NPs ratios, our results showed that NPs can carry plasmid DNA efficiently at saturating concentrations.

Next, we used HUVEC cells as endothelial cell model to evaluate the transfection efficiency of DNA encapsulated NPs. The transfected cells were analyzed by flow cytometry after transfection using NPs carrying p2X-GFP plasmid. GFP expression was detected in ~26% of the cells, indicating NPs/DNA can successfully transfect HUVEC cells. Next, we evaluated the efficacy of different sgRNAs for *in vivo* gene editing of mutant FVIII gene by hydrodynamic injection of Cas9/sgRNA plasmid into HemA mice. An immunodeficient hemophilia A (NSG HA) mice that contained premature stop codon in exon 1 of FVIII were used as the HemA mouse model. Two different sgRNAs that can edit wild type FVIII sequence (mF8sgRNA) or mutant FVIII exon 1 sequence (NSGHAsgRNA) were designed, respectively. Efficiency of gene editing was estimated *in vitro* by T7E1 assay using mouse embryonic fibroblast NIH3T3 cells. The results showed that mF8sgRNA can specifically induce double-strand breaks in wild type FVIII gene of NIH3T3 cells. The Cas9/sgRNA plasmids were hydrodynamically injected to NSG HA mice and the FVIII expression was examined by aPTT assay after one week. Both FVIII-targeting sgRNAs can promote the recovery of FVIII expression in NSG HA mice, suggesting the successful gene editing in mutant FVIII. After injection of Cas9/NSGHA sgRNA expression plasmid, FVIII activities maintained at least one month after treatment. Our data showed high transfection efficiency of DNA encapsulated NPs in HUVEC cells. Furthermore, we investigated *in vivo* gene editing using CRISPR/Cas9 technology to correct the mutated FVIII gene and regain the expression of FVIII protein in NSG HA mice. Our future goal is to use NPs that carry Cas9/sgRNA plasmid to correct the mutant FVIII gene in NSG HA mice.

Targeted Gene and Cell Therapy for Cancer

113. Gene-Based Immune Reprogramming Overcomes the Immunosuppressive Microenvironment of Liver Metastases and Enables Protective T Cell Responses

Thomas Kerzel[1], Federica Pedica[2], Stefano Beretta[1], Eloise Scarmadella[1], Tamara Canu[3], Rossana Norata[2], Lucia Sergi Sergi[1], Marco Genua[1], Renato Ostuni[1], Anna Kajaste-Rudnitski[1], Antonio Esposito[3], Masanobu Oshima[4], Francesca Sanvito[1], Mario Leonardo Squadrito[1], Luigi Naldini[1]

[1]San Raffaele Telethon Institute for Gene Therapy, Milano, Italy,[2]IRCCS San Raffaele Hospital, Milano, Italy,[3]Experimental Imaging Center, San Raffaele Scientific Institute, Milano, Italy,[4]Division of Genetics, Cancer Research Institute,

Kanazawa University, Kanazawa, Japan

The liver hosts an immune suppressive environment, which favors metastatic seeding and proliferation of cancer cells. Current pharmacological treatments, including most recent immunotherapies, fail in the presence of liver metastases (LMS). Therefore, identifying new interventional tools to break tumor tolerance and unleash immune responses in patients with LMS is of

[1] SR-TIGET San Raffaele Telethon Institute for Gene Therapy, Milano, Italy,[2]VitaSalute San Raffaele University, Milano, Italy,[3]German Cancer Research Center

(DKFZ), Heidelberg, Germany

Background: The pleiotropic immunostimulatory effects of cytokines such as interferon alpha (IFN-α) have been extensively investigated in cancer. However, systemic administration leads to sharp oscillations in cytokine plasma levels and OFF-target toxic effects, which strongly